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Full Length Research Paper

Comparative study of the antifungal activity of some essential oils and their major phenolic components against *Aspergillus niger* using three different methods

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This study aimed to evaluate the antimould activity of oregano, thyme, rosemary and clove essential oils and some of their main constituents: eugenol, carvacrol and thymol against *Aspergillus niger*. This antifungal activity was assessed using broth dilution, disc diffusion and micro atmosphere methods. In both agar diffusion and micro atmosphere tests, all the investigated agents showed no inhibitory effect on *Aspergillus niger* growth at concentrations lower than 10% (v/v). However, broth dilution test showed the highest sensitivity. Using this method, the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values of the tested agents were between 0.025 and 1%. The anti-*Aspergillus* effect of oregano and thyme oils was more potent than that of clove and rosemary oils. Concerning the phenolic compounds, thymol and carvacrol proved to have better anti-*Aspergillus* effect than eugenol. Accordingly, we can say that the antifungal efficacy of these agents is better appreciated when they are applied directly into liquid medium than when they are applied as volatiles or diffused in solid medium. Therefore, as these agents are active at low concentrations, they could be used in the formulation of natural preparations, and thereby could be proposed in therapeutic or hygienic contexts.

Key words: Essential oils, thymol, carvacrol, antifungal activity, *Aspergillus niger*, micro atmosphere, agar diffusion, broth dilution.

INTRODUCTION

Aspergillus niger (*A. niger*) is a ubiquitous filamentous fungus found in grains, fruits, forage, mouldy vegetables and dairy products. *A. niger* is a toxinogenic specie that can produce ochratoxin A (Abarca et al., 1994). It is also a pathogenic mould that causes otomycosis and aspergillosis (Araiza et al., 2006; Bulpa et al., 2007). The antifungal (anticandidal or antimould) effect of the essential oils (EOs) has been described in several studies (Knobloch et al., 1989; Arras and Usai, 2001; Pina-Vaz et al., 2004; López et al., 2007). These studies showed that some EOs have an inhibitory effect on the growth of fungi. Furthermore, it has been shown that the inhibitory effect of these EOs is due to the presence of the phenolic major components (Boonchird and Flegel, 1982; Viollon and Chaumont, 1994; Manohar et al., 2001). The majority of these researches have studied the antifungal effect on yeasts such as *Candida albicans* and *Cryptococcus neoformans*. However, only a small number of works have studied the anti-*Aspergillus* activity of the EOs and their main components. In addition, the studies concerning the antimould activity of the EOs have been conducted using the broth dilution method (Lambert et al., 2001; Pinto et al., 2006), the agar diffusion method (Cowan, 1999; Kosalec et al., 2005) or the micro atmosphere method (Paster et al., 1995; Inouye et al., 2000). The antimould volatile property of the major pure constituents of the EOs has been rarely reported (López et al., 2007). Moreover, the data obtained by these

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studies are not comparable due to the difference between the various methods.

The aim of the present work was to compare the antimould effect of oregano, thyme, rosemary and clove EOs and some of their major phenolic components, eugenol, carvacrol and thymol against *A. niger*, using three different methods: broth dilution, agar diffusion and micro atmosphere. This study is an attempt to select the best test for the evaluation of the EOs antifungal activity. This could be exploited in developing some hygienic or therapeutic preparations. These preparations could be useful against postharvest pathogens, for the disinfection of surfaces or air, and for the treatment or prevention of otomycosis and aspergillosis.

MATERIALS AND METHODS

Antifungal agents

The EOs used in this work were: oregano oil (*Oreganum compactum*) containing 35% of carvacrol and 25% of thymol, thyme oil (*Thymus vulgaris*) containing 62% of thymol, rosemary oil (*Rosmarinus officinalis*) containing 43% of 1, 8- Cineol. These EOs were purchased from Florarome (Fez, Morocco). Clove oil (*Eugenia caryophylata*) containing 90% of eugenol was provided by Osi (France). Carvacrol, thymol and eugenol used in this study were purchased from Fluka (Steinhein, Germany). These EOs and phenolic compounds were dispersed in 0.2% sterile agar suspension according to the method developed by Remmal et al. (1993a). The tested concentrations ranged from 0.01 to 0.1%.

Fungal strain

The fungal strain used in this work was *Aspergillus niger* (HA37). It was isolated from the Olive mill waste water and identified by standard methods of microbiology (Aissam et al., 2005).

Culture medium

Malt extract agar (Biokar) with chloramphenicol (Sigma) was used for fungus culture and maintenance. Malt extract broth was used to determine the minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC).

Inoculum preparation

The suspension of spores was prepared by growing *A. niger* on Malt extract agar at 30°C until sporulation. Some spores were then detached and transferred in a tube containing sterile NaCl 0.9%. This suspension was counted in Malassez counting chamber and diluted to obtain a final inoculum of approximately 10^6 spores/ml.

Micro atmosphere method

The following method allows studying the effect of the volatile fraction of the EOs and their major components. This test was performed in sterile Petri dishes (85 mm diameter) containing 20 ml of malt extract agar (MEA) medium. After spreading 100 µl of spore suspension (10^6 spores/ml) over the surface of malt extract, the Petri dishes were overturned. Then, a sterilized filter paper disc was placed in the centre of the cover of limps soaked with various concentrations ranging from 0.1 to 10% of the tested agents (Maruzzella and Sicurella, 1960). The disc used for the control contains dilution solvent (agar 0.2%). The plates were then tightly sealed with parafilm and incubated at 30°C. The dishes were observed every 24 h until growth was observed in the control subculture. The results were then estimated as the mean diameter of clear zones above each disc.

Agar disc diffusion method

This test was performed in Petri dishes (85 mm diameter) containing 20 ml of MEA medium. A sterile paper disc was placed on the surface at the centre of agar plates previously inoculated with a fresh fungal suspension (10^6 spores/ml). Subsequently, 100 µl of EOs (oregano, thyme, rosemary or clove), or phenolic compounds (carvacrol, thymol or eugenol), were applied on each paper disc at concentrations ranging from 0.1 to 10% (Bauer et al., 1966). The control disc contained only agar at 0.2%. The agar plates were then incubated at 30°C until growth was observed in the control subculture. The results were recorded by measuring the zones of growth inhibition around the discs.

Broth dilution method

Determination of the minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC)

The MIC determination was conducted in triplicate in a liquid medium by direct exposure of *A. niger* spores to increasing concentrations of the tested antifungal agents according to the method described by Remmal et al. (1993a), and incubated at 30°C for 6 days. The MIC was defined as the lowest antifungal agent concentration at which there was a visual absence of growth compared to that produced by the growth control tube. In order to evaluate the MFC, fractions of 20 µl from the tubes showing no growth were aseptically transferred into new tubes containing 980 µl of sterile malt extract. After an incubation period of 6 days at 30°C, the tubes were examined. The MFC was defined as the lowest antifungal agent concentration at which there was a visual absence of growth.

RESULTS AND DISCUSSION

Micro atmosphere test

The results of the antifungal effect of the studied EOs and some of their major components by micro atmosphere test are shown in Table 1. At concentrations of 1, 0.2 and 0.1%, no inhibition zone was obtained for all the tested agents. However, these agents at 10% (v/v) show an antifungal activity against *A. niger* with inhibition zones from 40 to ≥ 85 mm. Concerning the EOs, the largest inhibition zone was observed with oregano (≥ 85 mm). Thyme and clove oils show moderate effect with inhibition zones of 70 and 40 mm, respectively. No activity was noticed with rosemary oil. Among the tested phenolic compounds at 10%, thymol gave a larger inhibition zone (≥ 85 mm) than carvacrol (56 mm) and eugenol (49 mm). These findings agree with other studies reported by Guynot et al. (2003) and López et al. (2007). Several

Table 1. Antifungal activity of the tested EOs and phenolic components on *A. niger* by micro atmosphere test.

Essentials oil	Inhibition zone (mm)				
	10% (v/v)	1% (v/v)	0.2% (v/v)	0.1% (v/v)	Control
Rosemary	-	-	-	-	-
Thyme	70	-	-	-	-
Oregano	≥85	-	-	-	-
Clove	40	-	-	-	-
Phenolic component					
Carvacrol	56	-	-	-	-
Thymol	≥85	-	-	-	-
Eugenol	49	-	-	-	-

- : No inhibition zone was observed, *A. niger* grew even in front of the disc.

Table 2. Antifungal activity of the tested EOs and phenolic components on *A. niger* by agar diffusion test.

Essentials oil	Inhibition zone (mm)				
	10% (v/v)	1% (v/v)	0.2% (v/v)	0.1% (v/v)	Control
Rosemary	-	-	-	-	-
Thyme	56	-	-	-	-
Oregano	≥85	-	-	-	-
Clove	45	-	-	-	-
Phenolic component					
Carvacrol	≥85	-	-	-	-
Thymol	≥85	-	-	-	-
Eugenol	52	-	-	-	-

- : No inhibition zone was observed, *A. niger* grew even over the disc.

investigations showed that the antifungal activity of the volatile compounds by vapour contact is better than that obtained by broth dilution or by agar diffusion (Inouye et al., 2000; Suhr and Nielsen, 2003). The results obtained in our experimental conditions showed that the antifungal effect of the EOs and their major components by vapour contact are limited to high concentration. Even if this method is easy to use and widely utilised, it is principally a qualitative test which gives no more than an idea about the volatile fraction of the EOs. The results of the micro atmosphere method could therefore be useful for the development of some preparations used as fumigants applied in food, veterinary and medical fields.

Agar diffusion test

The results of the agar diffusion test are shown in Table 2. These results indicate that the studied EOs and phenolic compounds produced an inhibition diameter from 52 to ≥ 85 mm at concentration of 10% (v/v). Oregano oil with inhibition zone ≥ 85 mm was the most effective one, followed by thyme and clove oils with an inhibition diameter of 56 and 45 mm, respectively. It was also observed that rosemary oil has no effect on *A. niger* in this test. The anti-*Aspergillus* activity of eugenol, with inhibition zone of 52 mm, is weaker than that of carvacrol and thymol (≥ 85 mm). At concentrations lower than 10%, no inhibition zone was obtained with all the tested agents. Concerning the agar diffusion method, it has been routinely used in antibacterial susceptibility testing. However, the substances normally tested by this method such as antibiotics are generally hydrophilic. In contrast to antibiotics, EOs contains hydrophobic components which do not diffuse easily in the agar medium. It has been reported that the large inhibition observed with some EOs is not only due to the effect of their diffusion, but it is also due to the vapour effect of these oils in the agar medium (Inouye et al., 2006). In addition, the scientists, who have evaluated the antimicrobial activity of the EOs by the agar diffusion test, have frequently dispersed EOs in detergents and solvents such as dimethyl sulfoxide (DMSO) (Sabulal et al., 2006), ethanol (Bergonzelli et al., 2003) and Tween 80(Inouye et al., 2006). These dispersing agents increase the EOs diffusion in the agar medium, but they have some

Table 3. MIC and MFC of the tested EOs and phenolic components on *A. niger* by broth dilution test.

Essentials oil	MIC% (v/v)	MFC% (v/v)
Rosemary	0.4	1
Thyme	0.05	0.1
Oregano	0.05	0.05
Clove	0.1	0.1
Phenolic component		
Carvacrol	0.025	0.05
Thymol	0.025	0.025
Eugenol	0.05	0.1

drawbacks. In the present study, EOs and their major components were dispersed in an agar viscous solution (0.2%) without using any detergent or solvent. It has been previously demonstrated (Remmal et al., 1993a, b) that the dispersing agents habitually used (Tween 80, TritonX-100 and ethanol) have an inhibitory effect on the antimicrobial activity. This inhibition was confirmed later by other authors (Hili et al., 1997; Inouye et al., 2001). Therefore, this widely used test does not allow accurately measure of the antifungal activity of the EOs and their major components; it is a qualitative method which can be used as a preliminary test to select efficient EOs.

Broth dilution test

The MIC and MFC values obtained with rosemary, thyme, oregano and clove EOs, and with some of their major components, carvacrol, thymol and eugenol against *A. niger* are summarized in Table 3. According to these results, it seems that oregano (MIC = MFC, 0.05%) and thyme (MIC = 0.05% and MFC = 0.1%) oils are more efficient than clove (MIC = MFC, 0.1%) and rosemary oils (MIC = 0.4% and MFC = 1%) against *A. niger*. These results also show that the *A. niger* growth is completely inhibited by carvacrol, thymol and eugenol at the concentration of 0.025, 0.025 and 0.05%, respectively. In addition to that thymol had better fungicidal activity (MFC = 0.025%) than carvacrol (MFC = 0.05%) and eugenol (MFC = 0.1%). Many publications have reported the antifungal activity of the EOs (Knobloch et al., 1989; Tantaoui-Elaraki and Beraoud, 1994; Manohar et al., 2001; Pina-Vaz et al., 2004). It has been demonstrated that this property is essentially due to the presence of some major phenolic components such as thymol, eugenol and carvacrol (Inouye et al., 2001; Pina-Vaz et al., 2004; Segvić-Klarić et al., 2007).

Using the broth dilution method, an antifungal effect was noticed even at low concentrations (0.025%). The results of this test showed that oregano and thyme oils are more efficient against *A. niger* than clove oil. This is in agreement with what has been previously published using the same oils (Suhr and Nielsen, 2003; Pina-Vaz et al., 2004; Viuda-Martos et al., 2007). Rosemary oil showed a weaker activity on *A. niger* growth compared to oregano, thyme and clove oils. These findings parallel those already reported by Suhr and Nielsen (2003). The results we have obtained with the phenolic major components using the broth dilution method revealed that thymol and carvacrol have more potent anti-*Aspergillus* effect than eugenol, mentioning that in our experimental conditions, thymol (MFC = 0025%) had a better fungicidal effect than carvacrol (MFC = 005%). The discrepancy between the last results and those concerning the fungicidal effect of EOs would be explained by the fact that the oregano oil used in this work contains both carvacrol and thymol, while the thym oil contains only thymol. Several publications have previously reported that the antimicrobial activity of EOs is due to the presence of these major components (Knobloch et al., 1989; Dorman and Deans, 2000; Pina-Vaz et al., 2004). The MIC and MFC values obtained in this work were similar to those reported by Pinto et al. (2006). Qualitative comparison of the data obtained with the three methods revealed that the results of the agar diffusion and micro atmosphere assays agree with those obtained with the broth dilution assay concerning the efficacy of the EOs and phenolic compounds. The difference between the three methods is essentially quantitative. The antifungal activity of these agents in the micro atmosphere method and agar diffusion method is limited to high concentrations, while the broth dilution method is more sensitive and the anti-*Aspergillus* effect is observed at very low concentrations. So, in our experimental conditions the anti-*Aspergillus* efficiency of these agents was better appreciated when they were applied directly into liquid medium than when they were applied as volatiles or diffused in solid medium. These results are in agreement with those obtained by Suhr and Nielsen (2003).

Conclusion

Both volatile and diffusion tests should be used as preliminary tests when screening for determining the anti-

Aspergillus potential of the EOs, which would lead us to use the broth dilution method to accurately reveal the MIC and the MFC values. This will help to elaborate new formulations of natural preparations exploited in hygienic and therapeutic contexts. For example, we have managed in our laboratory to show the fungicidal activity of eugenol and carvacrol *in vivo* against *Candida albicans* on oral and vaginal experimental candidiasis in rats model (Chami et al., 2004a, b).

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